Development of a novel screen to dissect Toxoplasma gondii egress

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DEVELOPMENT OF A NOVEL SCREEN TO DISSECT *TOXOPLASMA GONDII* EGRESS

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Abstract

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By Keith Eidell
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The Apicomplexa comprise a group of obligate intracellular parasites some of which cause severe diseases in humans with malaria the most notorious representative. *Toxoplasma gondii* infection is the most widespread apicomplexan infection, which is mostly symptomless in healthy people but is associated with a variety of birth defects upon congenital infection and can become life threatening in immunocompromised patients. In addition, *T. gondii* has been established as a model for the study of intracellular parasitism by Apicomplexa. The lytic destruction of host cells underlies the pathogenesis of all apicomplexan diseases. The *T. gondii* lytic cycle involves host cell invasion, several rounds of intracellular replication, and is followed by egress of motile parasites in order to infect neighboring host cells. Egress is an increasingly more appreciated aspect of the lytic cycle for which three physiological triggers have been identified. All three triggers converge on the release of Ca^{2+} stores within the parasite. Large sections of the signaling pathways and molecular players associated with egress and intracellular calcium release remain unknown.

The objective of this thesis was to develop and employ a novel enrichment screening procedure that would efficiently isolate egress mutants in response to pharmaceutically induced egress. The biggest caveat to such a screen is the ability to separate intracellular from extracellular parasites, which is hampered by the stickiness of

parasites to host cells as well as their fast reinvasion capacity. This hurdle was overcome by saturating the parasite's surface receptors with the glycan heparin to prevent attachment to the host cell. Simultaneously, the oxidizing agent pyrrolidine dithiocarbamate (PDTC) was applied to specifically kill extracellular parasites. The enrichment power of the screen was assessed by diluting a previously identified temperature-sensitive egress mutant called F-P2 in wild type parasites. The screen's enrichment power was assessed by flow cytometry and a 1000-fold enrichment capacity to a 100% F-P2 population could routinely be achieved. Subsequently the screen was applied to generate mutants with defects in the poorly understood NTPase mediated egress-trigger pathway. Chemical mutagenesis as well as insertional mutagenesis was applied and dithiotreitol (DTT) that artificially creates the reducing environment triggering egress was used to screen mutants. Three chemically induced constitutive egress mutants and one insertional mutant were isolated. As expected, all mutants displayed resistance to DTT induced egress. In addition, cross resistance to two other egress inducers upstream of Ca^{2+} release was observed, however all mutants egressed upon calcium ionophore treatment.

Taken together, the developed enrichment procedure will enable the isolation of constitutive as well as conditional egress mutants. Future cosmid complementation will help to fill in important blanks in the egress mechanisms and will ultimately lead to a better understanding of intracellular parasitism. This gained understanding will potentially lead to therapies to combat the destructive effects of apicomplexan parasites.

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List of Abbreviations

ABA abscisic acid
ATP adenosine triphosphate
BAPTA-AM 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
Ca^{2+} calcium
$CaCl_2$ calcium chloride
cADPR cyclic adenosine 5'-diphosphate ribose
$CaHCO_3$ calcium carbonate
CAT chloramphenicol acetyltransferase
CDPK calcium dependent protein kinase
CSA chondroitin sulphate A
CSC chondroitin sulphate C
CTL cytotoxic T lymphocyte
DHFR dihydrofolate reductase
DNA deoxyribonucleic acid
DS dextran sulphate
DTT dithiotreitol
EMS ethyl methanesulfonate
ENU *N*-ethyl-*N*-nitrosourea
FasL Fas ligand
GFP green fluorescent protein
HA haemagglutinin
HFF human foreskin fibroblast
HXGPRT hypoxanthine xanthine guanine phosphoribosyl transferase
IL interleukin
IP_3 inositol 1,4,5-trisphosphate
K^+ potassium
$MgCl_2$ magnesium chloride
NTPase nucleoside triphosphate hydrolase
NK natural killer
NH_4Cl ammonium chloride
PBS phosphate buffered saline
PIP_2 phosphatidylinositol 4,5-bisphosphate
PLC phospholipase C
PDTC pyrroilidine dithiocarbamate
RFP red fluorescent protein
TNF-α tumor necrosis factor alpha
YFP yellow fluorescent protein

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Chapter 1. Introduction.

1.1 Apicomplexans

Several organisms such as *Toxoplasma, Plasmodium, Cryptosporidium* and *Neospora* are example members of the phylum Apicomplexa. The aspect that defines all these organisms is their apical complex. The apical complex consists of several apical secretory organelles along with a very apical localized tubulin-based structure termed the conoid with anteriorly and at its base several circular structures named polar rings. These unicellular pathogens are obligate intracellular parasites. They reside intracellularly within a host cell and utilize the host to their own benefit (there is no extracellular replication). Hence, the success of a parasite requires that the pathogen not kill the host and to not be destroyed by the host's immune system. The apicomplexans are particularly of interest due to the physical symptoms they can cause (e.g. diarrhea, flu like symptoms) with death as the most extreme outcome as in the case of *Plasmodium* (the causative agent of malaria). Clinical disease caused by *Toxoplasma* (toxoplasmosis) typically is limited to immunocomprised individuals and developing fetuses. Some of the clinical manifestations associated with toxoplasmosis are encephalitis, myocarditis and birth defects [1,2,3].

1.2 Toxoplasma gondii General Biology

A typical *Toxoplasma gondii* parasite is about 10-12 µm in length and 2-4 µm in width. The parasite contains a nucleus, a single mitochondrion, endoplasmic reticulum, a

calcium rich storage organelle termed the acidocalcisome and the secretory dense granules. At the apical end the parasite contains cigar shaped secretory organelles called micronemes as well as club shaped organelles called rhoptries. A non-photosynthetic, chloroplast-related organelle called the apicoplast is located at the anterior end of the nucleus and is associated with the lipid metabolism of the parasite and carries its own 35 kb circular genome (Figure 1) [1].

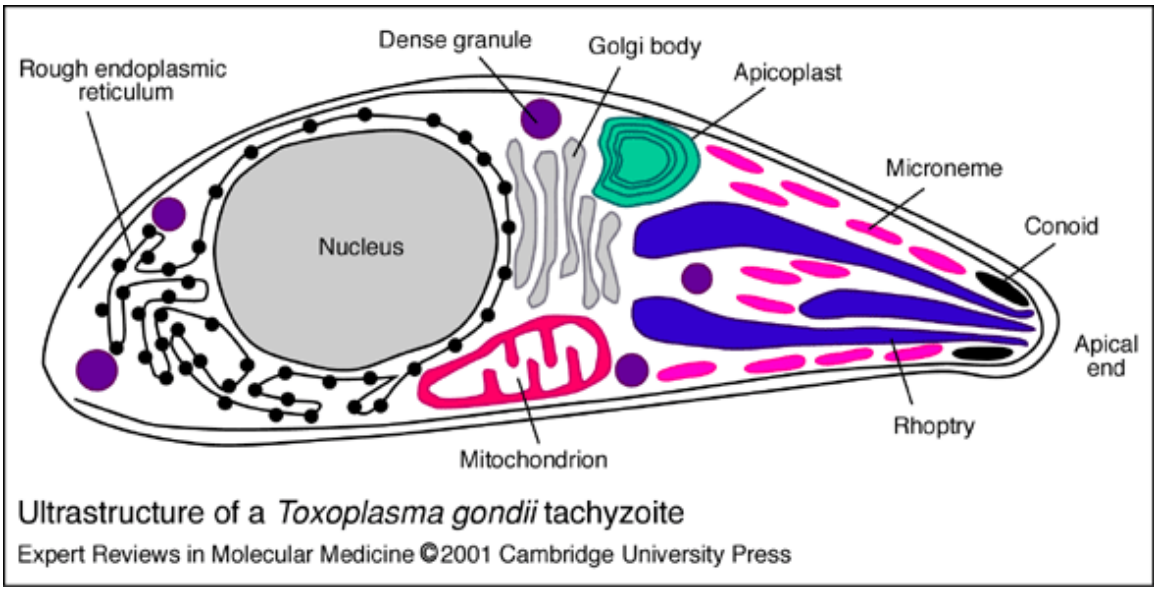

Figure 1. *Toxoplasma gondii* parasite organelles. The cigar-shaped microneme organelles, the club-shaped rhoptry organelles and the chloroplast-like organelle called the apicoplast are all apical. The parasite also contains dense-granules throughout the cytoplasm. In addition the parasite contains a single mitochondrion, endoplasmic reticulum, Golgi apparatus and nucleus. Illustration used with permission from Cambridge University Press through RightsLink Copyright Clearance Center, license 2544980833495.

The life cycle of *T. gondii* involves sexual replication in the intestine of a cat (definitive host) as well as asexual replication in nucleated cells from any warm-blooded animal (intermediate hosts e.g. human) [1,2,3]. In a feline the sexual cycle unfolds

through gametogenesis, zygote formation and oocyst formation. Oocysts, containing sporozoites, are shed in the feces of the cat and will become fully infectious in the environment. Asexually there are two stages; tachyzoites and bradyzoites. In the asexual life cycle tachyzoites can transition to bradyzoites, which can then transition back to tachyzoites [1,2,3]. Possible transmission of parasites occurs with a cat eating a mouse or bird that contains bradyzoite parasite cysts or with the intermediate (human) host consuming either undercooked meat containing bradyzoite cysts or vegetables or surface water contaminated with oocysts (cat litter). Upon ingestion cyst rupture will occur in the digestive tract releasing bradyzoites or sporozoites, which subsequently infect the small intestine epithelium and differentiate into tachyzoites which disseminate throughout all host tissues [3].

Bradyzoites have a very slow replication cycle and form tissue cysts. Within these cysts they can remain dormant for long, variable periods of time. Typically, upon infection tissue cysts will appear 7-10 days post-infection. Cysts of bradyzoites are found distributed within the central nervous system as well as in muscle tissue. A cyst typically has a thick wall of chitin and glycoproteins protecting the bradyzoites from the external environment. Additionally, the differentiation to bradyzoites includes reduction of the apicoplast as well as relocation of the nucleus to a more basal position in the parasite [4,5,6].

Differentiation from tachyzoites to bradyzoites occurs as a result of the emerging immune response upon infection (e.g. IL-12, TNF-α, interferon-γ). Cysts can be induced in the laboratory by environmental stresses such as alkaline pH, environmental CO_2

concentration [7], high temperature (heat shock) [7], proinflammatory cytokines [8], incubation with atovaquone [9,10] as well as by incubation with fluridone [11]. Development of bradyzoite cysts can be monitored through reactivity with the *Dolichos bifloris* lectin agglutin (staining for the cyst wall) or by using antibodies recognizing bradyzoite specific surface proteins [4,5].

The population structure of *T. gondii* is very clonal with three genotypes making up 90% of the worldwide population; genotypes not fitting in these three types are considered "exotic" and are mostly restricted to South America. The Type I strain is the most virulent strain in mice. The Type I RH strain, which is most commonly used in the laboratory to study the acute phase of tachyzoites in the lytic cycle, has lost the ability to develop into fully mature bradyzoites. In addition, the RH strain has lost its ability to infect cats. Other Type I strains have the ability to transition into both bradyzoites and infect the cat. A widely used Type II strain is ME-49 whereas VEG is a widely used Type III strain, which both can complete all stages of the *Toxoplasma* life cycle [1,2,3].

1.3 The Lytic Cycle of T. gondii

The lytic cycle of tachyzoites leads to the destruction of host cells in the replication process. All pathology is associated with its lytic cycle. In contrast to bradyzoites, tachyzoites have a very short replication cycle and do not form cysts. An essential step of this cycle is the invasion of the host cell. First an extracellular tachyzoite glides over the surface of a host cell and establishes a tight attachment. From here the tachyzoite will enter the host cell cytoplasm and form its parasitophorous vacuole by

invagination of the host cell's plasma membrane [12]. Several rounds of replication occur within the vacuolar compartment. Upon consumption of most of the host cell resources the tachyzoites egress and the next cycle will begin by invasion of neighboring host cells (Figure 2) [1]. Depending on the size of the host cell, typically once a vacuole contains 64-128 tachyzoites (36+ hours) they will egress and invade neighboring host cells [1].

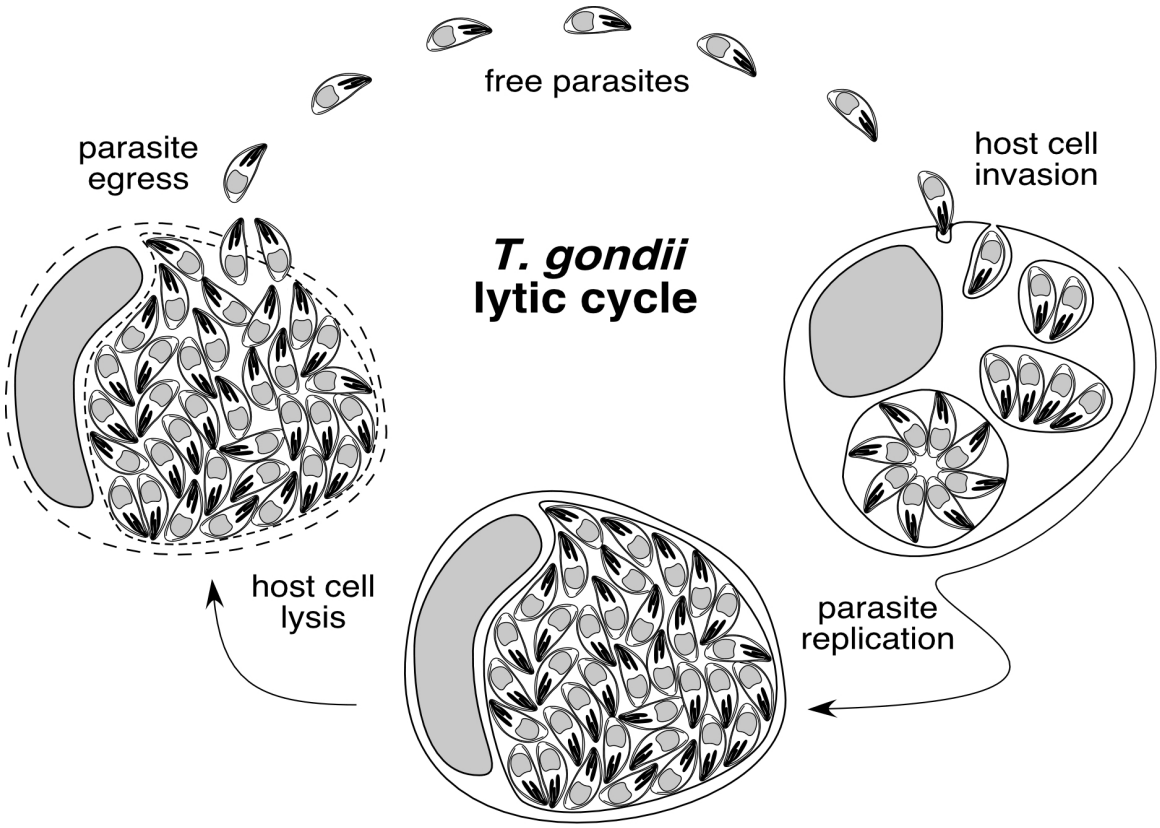

Figure 2. The Lytic Cycle of *Toxoplasma* tachyzoites. The lytic cycle consists of an extracellular parasite gliding over the surface of a host cell followed by invasion of the host cell by the parasite. Once residing intracellularly the parasites undergo several rounds of replication followed by egress of parasites from the host cell. Released tachyzoites are able to infect neighboring host cells. The lytic cycle of the *Toxoplasma* tachyzoite causes the destruction of host cells and is associated with all pathology.

1.4 T. gondii as a Genetic Model Organism

In order to prevent physical symptoms and fatalities caused by apicomplexan parasites *via* vaccines or drugs a better understanding of the overall biology governing their life cycles and biological processes is needed. To identify major events, molecular players and intricate signaling pathways involved in the life cycles of apicomplexans, experimental accessibility is required, which unfortunately is not the case for most Apicomplexa due to their complex and multi-host development cycles.

T. gondii makes a great model organism, most importantly due to the continuous tissue culture system of the tachyzoite life stage. Other advantages are the fast generation time, the availability of a sequenced and annotated genome and the high efficiency of transient and stable transfections allowing tagging (e.g. GFP, HA, MYC) and targeted knock-outs of genes. These tags can be of use for genetic, cell biological or biochemical studies [2,13].

The features discussed above fulfill the requirements of a genetically tractable organism and consequently forward genetic approaches comparable to other genetic models such as yeast, worm and fruit fly have been developed. An important recent milestone was the development of technology to genetically complement mutant phenotypes since genetic crosses are not feasible with the RH lab strain [14]. Genetic complementation is based on wild-type genomic DNA cosmid libraries that enable the throughput to gene identification of performing forward genetics on the parasite [14].

Generating mutant phenotypes of *Toxoplasma* without a prior assumption on the nature of the gene is through random mutagenesis. Two forms of mutagenesis can be applied; the first is insertional mutagenesis. Insertional mutagenesis involves the

transfection of an exogenous linearized vector that will randomly integrate into the genome through non-homologous recombination [15]. Applying this type of mutagenesis targets mostly non-essential genes. Phenotypes generated from this type of mutagenesis are constitutive and non-lethal.

Chemical mutagenesis is the second type that can be applied. Applying this particular type of mutagenesis enables the targeting of essential genes by selecting for conditional (e.g. temperature sensitive phenotypes) [13,16,17]. Mutant phenotypes associated with this type of mutagenesis can be conditional at the restrictive condition but behave as wild-type at the permissive condition (e.g. high versus low temperature). Constitutive mutant phenotypes can also be generated through chemical mutagenesis. Conditional phenotypes are much more evident of essential genes than constitutive phenotypes since some conditional phenotypes lead to lethality at the restrictive condition. Identifying the mutated genes from an insertional mutagenesis involves rescue of the insert as well as a piece of flanking genomic DNA [15]. Identifying mutated genes from a chemical mutagenesis involves cosmid complementation followed by rescue of part of the complementing cosmid genomic DNA library insert [14].

Besides forward genetics, several genetic approaches to conditionally express genes are available in *T. gondii* for reverse genetic (e.g. candidate gene-based) studies. Since the parasite has a haploid genome these are important technologies enabling the study of pre-selected essential genes. The use of a regulatable promoter utilizing addition of tetracycline has become a standard method [18]. An ulterior technology for the conditional expression of genes makes use of a fusion of a ligand-controlled

destabilization-domain that is regulated by the addition of a small molecule termed Shield-1. Upon the addition of Shield-1 whatever protein is fused to the destabilization domain will become stabilized and expressed. In absence of Shield-1 proteins are degraded immediately by the proteasome. These particular techniques have enabled the ability to perform reverse genetics on essential genes [19,20].

1.5. T. gondii Host Cell Invasion

The process of host cell invasion lies at the heart of intracellular parasitism. Overall invasion of host cells involves a number of successive steps [12] (Figure 3). In contrast to many other intracellular pathogens, apicomplexan cell invasion is completely controlled by the parasite and not dependent on phagocytic mechanisms of the host, which explains why cells other than macrophages can be host cells. Host cell invasion mechanisms are widely conserved across the Apicomplexa. The choice of host cells differs per parasite and depends on recognition of specific receptors on the surface of the host. In the case of *Toxoplasma*, these are generic proteoglycans found on all nucleated cells [21,22]. Initial recognition of the host cell is mediated by GPI-anchored antigens on the surface of the parasite (SAGs). TgSAG1 and TgSAG3 have been found to be involved in initial attachment by tachyzoites [23,24].

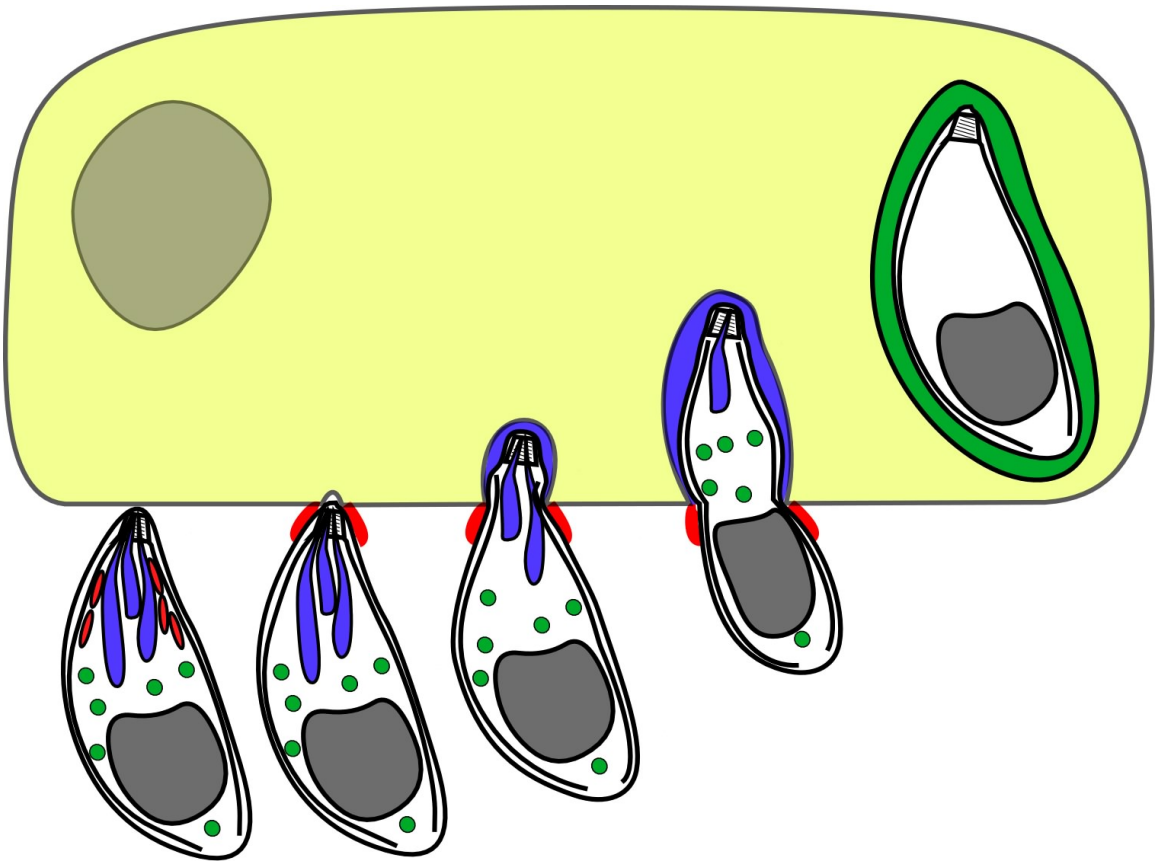

Figure 3. The process of parasite host cell invasion. Once an extracellular parasite glides over the surface of a host cell, initial attachment is mediated through GPI-anchored surface antigens (SAGs). Stronger attachment is mediated through secretion of microneme organelle proteins that bind to host cell surface receptors (red). Rhoptry proteins (purple) are then secreted and form a moving junction at the site of parasite invasion that excludes host cell surface proteins as well as contribute to intracellular vacuole membrane development. Dense granules (green) are secreted last and form a tubulovesicular network in the vacuole involved in nutrient acquisition [12].

1.5.1 The Micronemes

Once initial contact has been made the parasites will secrete their micronemes mediated by release of intracellular Ca^{2+}. Micronemes, contain adhesion type proteins that aid the tachyzoite in making a stronger attachment to the host cell by binding to additional host cell surface receptors. Many of the MICs are secreted as complexes such as TgMIC2 and an accessory protein (TgMIC2-TgMIC2-AP) and as a complex of

TgMIC4, TgMIC6 and TgMIC1 [25]. TgMIC2 and TgMIC6 associate also with aldolase, which is able to interact with actin [25,26]. Through KO-studies MIC proteins have been found essential for invasion (TgMIC2, TgMIC8, TgAMA1) [27,28,29]. Successful MIC secretion requires packaging of contents into vesicles involving the vesicular scaffolding protein TgPRP1 [30]. Microneme proteins are secreted at the very apical end of the parasite, then anchor in the parasite's plasma membrane and are transported posteriorly over the surface of the tachyzoite [25,31]. Once they reach the basal end the microneme proteins are released by rhomboid proteases [31,32,33]. Besides upon shedding, microneme proteins are proteolytically processed at several points throughout the Golgi-mediated secretory pathway [32,33].

1.5.2 The Conoid

The conoid is an apical structure made up of tubulin, which extrudes upon release of intracellular Ca^{2+}. Once microneme secretion occurs the conoid retracts inwardly [34,35]. Although the exact role of the conoid is not well understood, one model is that the conoid functions as a jackhammer releasing the intracellular host cell matrix from the plasma membrane to generate the initial invagination required for the formation of the parasitophorous vacuole.

1.5.3 The Rhoptries

After microneme secretion and forming a tight contact point at the invaginated host cell plasma membrane, the parasite releases its rhoptry organelles. Like the

micronemes, the rhoptries are secreted at the very apical end of the parasite. Two different sub-regions of the club-shaped rhoptries are sequentially secreted. First the proteins residing in the neck of the rhoptries, termed RON proteins, are secreted. RON proteins such as TgRON2, 4, 5 and 8 together with one microneme protein (TgAMA1) establish a tight junction between the parasite and the host cell plasma membrane at the point of contact [36-38]. Subsequently the parasite starts moving further into the host cell. Since this junction remains at the plasma membrane while the parasite moves, this structure is known as the moving junction (MJ) [39].

Upon invagination of the host cell plasma membrane and traversing through the MJ, the rhoptry bulb proteins (ROPs) are secreted into the host cell through a mechanism resembling bacterial Type III secretion. Rhoptry proteins in vesicles termed evacuoles are directly injected into the host cell cytoplasm. Several of these proteins localize to the outside of the newly forming parasitophorous vacuole that envelopes the parasite [40], whereas others are signaling molecules interfering with many aspects of the host cell's physiology. One of the functions is to inactivate the host cells apoptotic mechanism to secure survival of the host throughout the duration of parasite replication [41].

1.5.4 Gliding Motility

As pointed out, host cell invasion is completely controlled by the parasite. In order to migrate to a host cell and to invade that cell the parasite therefore must contain a motility system. The common motility systems encountered across various organisms are absent in Apicomplexa; except from the male gametes no flagella are present and neither

are cilia. Furthermore, the parasites move without changing shape and therefore are not amoeboid. In contrast, the parasites are only able to move on solid substrate and display directed gliding motility, which is differentiated in gliding, helical or twirling movements [42]. Gliding makes use of a type XIV myosin (TgMyoA) as its motor and requires dynamic actin polymerization [43,44]. A conditional TgMyoA knock-out showed that myosin has an essential role in motility and invasion [45]. Subsequently it was shown that TgMyoA is part of a protein complex called the glideosome, which is anchored in the membrane cytoskeleton directly underlying the plasma membrane of the parasite known as the inner membrane complex (IMC). TgGAP50 is an integral membrane glycoprotein that is anchored within the IMC and is associated with another integral membrane glycoprotein TgGAP45. This so-called glideosome complex further contains a myosin light chain TgMLC1 [46-49].

Early drug studies showed that inhibiting actin polymerization disrupted motility and host cell invasion [46,44]. Apicomplexan actin is almost exclusively found in its G-form and very short actin filaments only form at the site of substrate contact in the narrow space between the IMC and the plasma membrane. Only very few actin associated proteins have been identified [50-54]. In order to power motility the actin filaments are anchored to the extracellular space through contact with the transmembrane microneme proteins. The C-terminal tail domains of MIC proteins (e.g.TgMIC2 and TgMIC6) have been found to associate with the glycolytic enzyme aldolase (TgALD1) on the cytoplasmic face of the plasma membrane. Aldolase in turn associates with F-actin

[55,56]. F-actin filaments are transported in an apical to basal direction by the action of TgMyoA enabling directed parasite movement (Figure 4) [46].

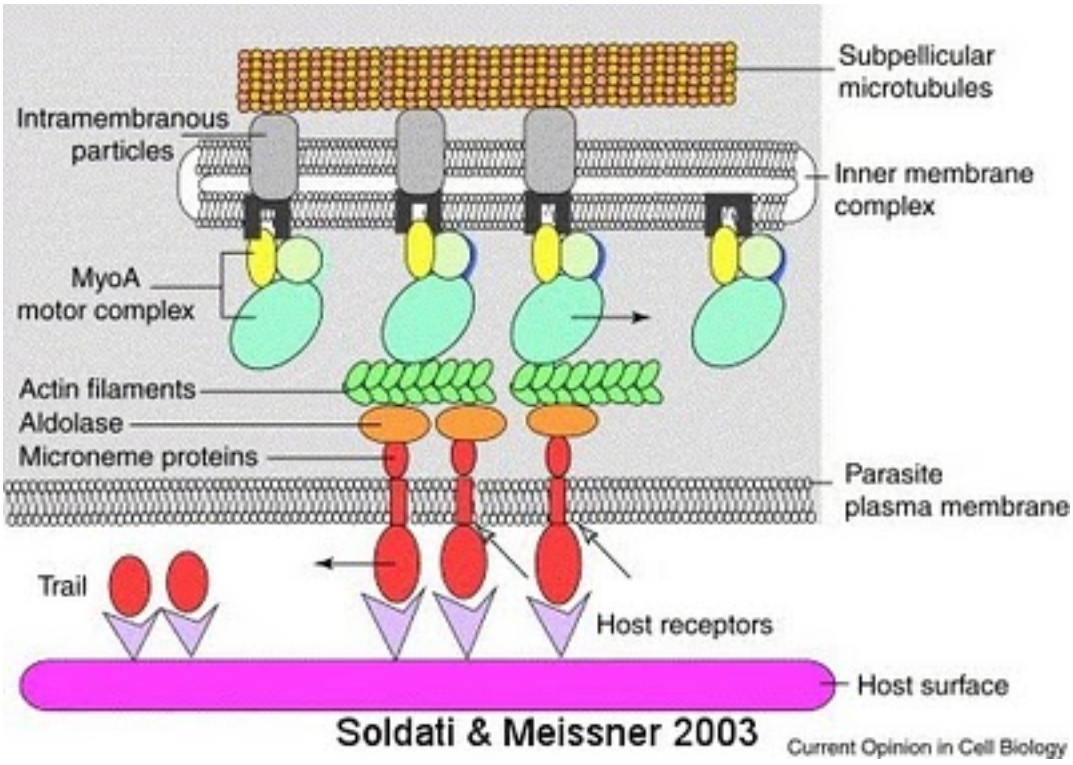

Figure 4. Glideosome complex that powers parasite motility. Model for the interaction between the myosin A motor complex and the micronemal protein–host receptor complex via aldolase/F-actin at the parasite pellicle. The black arrows indicate the movement of the complexes; the open arrows indicate the proteolytic cleavage by rhomboids [46]. Illustration used with permission from Current Opinion in Cell Biology through RightsLink Copyright Clearance Center, license 2544970786234.

1.6 T. gondii Egress

One very critical and increasingly more appreciated aspect of the lytic cycle is the event of tachyzoite egress from a mature vacuole, which is now considered the first step in the ability to infect neighboring host cells. One of the events associated with egress is the transition from an immotile, intracellular form into a motile extracellular form. The event of egress shares many mechanisms with the invasion aspect of the lytic cycle

involving intracellular Ca^{2+} release, gliding motility and microneme secretion. However, in some aspects egress is distinct (e.g. rhoptry secretion is necessary for invasion but not for egress) [57].

Upon maturation of a vacuole of immobile tachyzoites there are currently three physiological triggers recognized that lead to egress. The best understood mechanism is the loss of cytoplasmic K^+ concentration from the host cell upon plasma membrane rupture due to mechanical stress or the immune response [58,59]. The second trigger for egress is the spiked production of abscisic acid (ABA) produced by the parasite's plastid through a quorum sensing mechanism just prior to the event of egress. Addition of exogenous ABA stimulates MIC secretion of extracellular parasites and also artificially stimulates egress of approximately 7% of vacuoles in a given monolayer [11]. The third trigger of egress involves a family of nucleoside triphosphate hydrolases (NTPases) which are secreted into the vacuolar lumen through the dense granules [60]. Development of a reducing environment in the vacuole, likely upon host cell ATP depletion, activates the vacuolar NTPases and causes rapid egress of tachyzoites [61]. This third trigger for egress is the least understood mechanism.

Evidence is mounting that the host's immune system can also more directly cause parasite egress. Death receptor ligation through binding of Fas ligand (FasL) to infected cells or exposure to perforins produced by NK or CTL cells induces egress of tachyzoites. The effector T-cells are susceptible to infection and have been proposed as a mechanism contributing to dissemination of the parasites throughout the host [62].

In addition to the triggers of egress two major factors have been identified that facilitate egress in *Toxoplasma* once egress has been initiated. The first is a perforin-like protein TgPLP1 secreted through the micronemes that forms pores in the vacuolar membrane [63]. The second egress factor is the exploitation of host calpains that act on degrading the host cytoskeleton upon egress [64]. Individual KO studies of both egress factors showed vacuoles that popped-out of the monolayer without egressing.

1.7 Dissection of Signal Transduction in Invasion and Egress through Pharmaceutics

As described in the above section, the egress triggers need to be transduced into activation of the mechanisms required for egress such as microneme secretion, conoid extrusion, glideosome activation and actin polymerization. The biological context of the mechanisms underlying these signal transduction pathways have been dissected through the use of pharmaceuticals interfering with various signal transduction mechanisms. A critical aspect of all known triggers leading to egress is the release of intracellular Ca^{2+}.

Egress of vacuoles of tachyzoites can be artificially stimulated through the use of a variety of inducers such as Ca^{2+} ionophores [65,66], the K^{+} ionophore nigericin [67], the reducing reagent dithiotreitol (DTT) [68] and ethanol [69]. Cysts of bradyzoites on the other hand are not responsive to egress inducers, possibly due to their thick cyst wall [70]. Each inducer theoretically has different targets and hence parallel pathways leading to the convergence of intracellular Ca^{2+} release as well as targets after intracellular Ca^{2+} release can be dissected (Figure 5).

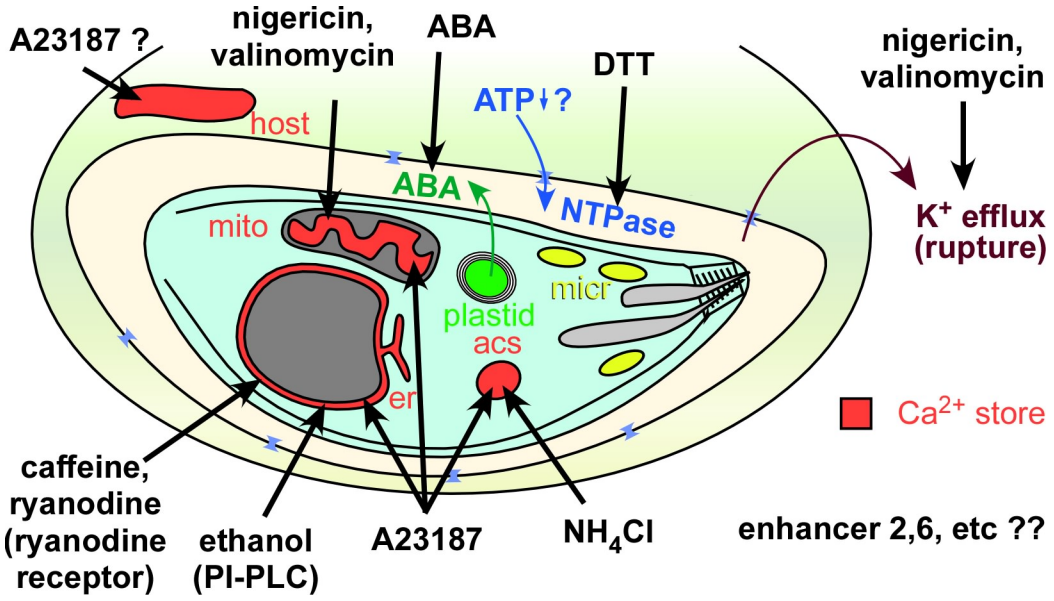

Figure 5. Ca^{2+} dependent egress triggers as well as pharmaceutical egress induction. Three physiological triggers lead to Ca^{2+} dependent egress (Ca^{2+} pools are in red). Loss of host cell cytoplasmic K^+ through membrane disruption or mechanical rupture (purple) is the most well understood mechanism [58]. Spiked production of abscisic acid (ABA) (green) from the plastid just prior to egress through a quorum sensing mechanism is the second trigger [11]. The least understood and third trigger involves the activation of vacuolar NTPases secreted through the dense granules (blue) following host cell ATP levels depleting [60,61]. Egress can be artificially induced through a variety of pharmaceuticals (all in black). Ca^{2+} ionophore A23187 can target any calcium rich organelle [65,66]. The reducing agent DTT targets the vacuolar NTPases [68] and ethanol targets the endoplasmic reticulum [69] as well as does ryanodine and caffeine [78]. The K^+ ionophore nigericin or valinomycin targets the mitochondria [67] and NH_4Cl targets the acidocalisome (acs) [73,83]. Ethanol targets phospho-lipase C (PI-PLC) on the ER membrane. Small molecule microneme and motility enhancers 2 and 4 have no known targets [88]. mito = mitochondria; micr = micronemes.

1.7.1 Utilization of Pharmaceuticals to Identify Calcium Release Channels

Calcium (Ca^{2+}) has been long found to be a central aspect of the tachyzoite lytic cycle [71]. Addition of ethanol, ammonium chloride (NH_4Cl), acetaldehyde or A23187 to extracellular tachyzoites cause spikes in intracellular Ca^{2+} concentration. Upon incubation with these intracellular Ca^{2+} release reagents microneme secretion occurs as well. Ca^{2+} chelators (e.g. BAPTA-AM) block Ca^{2+} release as well as microneme secretion [72,73]. Through the use of calmidazolium intracellular Ca^{2+} levels are elevated

as well as tachyzoites are found to have extended motility trails. The use of Ca^{2+} chelators inhibits the effect of calmidazolium [74]. Intracellular Ca^{2+} release has also been found essential for conoid protrusion [34,75]. Overall when intracellular Ca^{2+} release is inhibited with Ca^{2+} chelators invasion as well as egress are blocked [58,76].

The parasite contains 3 internal calcium stores (the mitochondrion, endoplasmic reticulum and the acidocalisome) that provide the calcium needed for microneme secretion and motility activation. Possible calcium release channels in the parasite include IP_3 and ryanodine sensitive stores. Ryanodine and caffeine stimulate Ca^{2+} release as well as MIC secretion in the parasite and the effect is inhibited with Ca^{2+} chelators. A sharp spike in IP_3 production was found upon addition of ethanol. Since IP_3 has been implicated in Ca^{2+} signaling the inhibitor Xestospongin C (inhibits IP_3 binding) was used and found to inhibit microneme secretion, attachment and invasion. The importance of phospholipase C (PLC) was shown with addition of the PLC inhibitor (U73122) that inhibited egress, as well as conoid protrusion [76-78]. This work builds a working model of Ca^{2+} signaling showing that phospholipase C (PLC) cleaves PIP_2 generating IP_3 that binds to IP_3 receptors releasing Ca^{2+} from the ER [76,78].

Like mammalian cells, *Toxoplasma* has ryanodine sensitive Ca^{2+}stores associated with an increase in the secondary messenger cADPR [79,80]. cADPR is produced by tachyzoites and stimulates intracellular Ca^{2+} release and MIC secretion. Antagonists of cADPR inhibit MIC secretion as well as motility [80]. Stress responses in plants involve cADPR signaling resulting from a spiked production in ABA [81]. In *Toxoplasma* cADPR increase results from a spiked production of ABA just prior to egress [11].

Just as it is important for Ca^{2+} to be released it is just as important that Ca^{2+} reuptake be regulated. Concurring with this model disruption of genes involved with Ca^{2+} pumping (e.g. TgSERCA, TgNHE1, TgA1) resulted in constitutively increased Ca^{2+} levels as well as displayed defects in invasion and egress [82-84].

Beyond the pivotal Ca^{2+} release signal lies a family of calcium-dependent protein kinases (CDPKs). When parasites are incubated with the CDPK inhibitor KT5926 attachment and egress are affected [85,58]. Through the use of a TgCDPK1-KO TgCDPK1 was shown to be directly responsible for exocytosis of microneme proteins. Without microneme exocytosis motility, invasion and egress are all affected [86].

1.7.2 Current Roadblock in Furthering Understanding of Egress Signaling Pathways

Although pharmaceuticals have been very helpful in identifying the kind of signaling pathways involved in egress, the exact molecular identity of many of the players (e.g. ligands and receptors) is currently not known. Several approaches have been used to identify these players. The first uses the sequenced genome as a guide to identify proteins in pathways sensitive to the drugs by sequence comparison. This approach has shown some validity in identifying candidate secretory proteins [87]. However, this approach has in many cases proven unreliable in its ability to identify new genes of interest. Particularly this approach falls short because there are several pathways identified by pharmaceuticals for which no clearly recognizable gene orthologs could be identified in the *Toxoplasma* genome (e.g. currently unknown IP_3 receptors targeted by ethanol [78]).

A second approach employs chemical genetics, which involves screening of small molecule libraries to identified compounds that act as inhibitors or enhancers of invasion, microneme secretion, conoid extrusion and motility [88]. Despite the shown usefulness of these compounds enabling more insight into the biology of the parasite (e.g. showing posttranslational modifications to the glideosome [89]) the identification of the actual protein targets of these compounds is lacking and is very challenging. Hence, this approach fails in the throughput and ability to identify new genes of interest involved in invasion and egress.

The third approach to unravel the mechanisms of egress is taking a forward genetic approach. This third approach requires the generation of egress mutants by subjecting parasites to mutagenesis followed by isolation of mutants from the mutagenized population. Once egress mutants are isolated either cosmid complementation or insert rescue is performed depending on the type of mutagenesis applied.

Application of artificial stimulation in order to dissect the signaling pathways underlying egress was shown by generation and isolation of egress mutants in response to A23187. Use of surface biotinylation of egressed parasites followed by column passage to remove biotinylated (egressed) parasites from unbiotinylated (intracellular) parasites that were of interest was used. Five rounds of this screening were performed followed by a secondary screen in which unresponsive vacuoles were individually picked [90]. The major caveats that rendered these screens inefficient are the several rounds and extensive

materials needed for mutant enrichment which originate in the stickiness and the fast-reinvasion capacity of tachyzoites [91].

1.8 My Approach to Study T. gondii Egress and Invasion

The objective of this thesis was to develop and implement a more efficient screening procedure for the enrichment and isolation of egress mutants in response to pharmaceutically induced egress. In addition it was aimed to design the screen to permit the isolation of the strongest phenotypes (e.g. to generate conditional mutant egress phenotypes). As discussed above, different classes of pharmaceuticals inducing egress work on different targets in the signaling pathway. Therefore it should theoretically be possible to generate a very diverse set of mutants using single inducers, or combinations of different inducers with or without subsequent differential enrichment rounds. Isolation of such mutants would help in answering the many remaining questions pertaining to Ca^{2+} signaling as well as unknowns in the initiation as well as regulation of egress. With the recently developed methodology to identify genes underlying conditional phenotypes, establishment of various egress mutants will provide the basis for future mapping of these genes and will generate insights in the molecular requisites involved in *Toxoplasma* egress and invasion. Since many aspects of these pathways are conserved across the Apicomplexa, I expect this approach will provide widely valid insights into the biology of the intracellular lifestyle of apicomplexan parasites.

Chapter 2. Materials and Methods.

2.1 Parasites and Host Cells

RH-strain parasites and derivatives were grown in human foreskin fibroblast (HFF) cells and transfected by plasmid electroporation as previously described [92]. 2F-1-YFP_2 parasites were described previously [93]. Cytoplasmic tandem TomatoRFP expressing parasites were generated by transfection of the tubTandem-TomatoRFP/sagCAT plasmid (kindly provided by Giel van Dooren [94]). The TgPLP1-KO parasite line expressing a construct that secretes dsRed-RFP into the vacuole was kindly provided by Vern Carruthers [63]. F-P2 parasites are mutant derivatives of the 2F-1-YFP_2 strain that was mutagenized with *N*-ethyl-*N*-nitrosourea (ENU) as previously described [14].

2.2 Insertional Mutagenesis

For insertional mutagenesis the plasmid ToxoSuperCos containing the DHFR-TSm2m3 selectable marker [14] was digested with *Bam*HI and *Nde*I to release the tandem Cos sites, gel-purified and transfected into RHΔHXGPRT parasites (derivatives of the RH strain). A stable transfected parasite population was selected by pyrimethamine selection for genomic integration of the ToxoSuperCos plasmid using 1 μM pyrimethamine.

2.3 Chemical Mutagenesis

For chemical mutagenesis 1 ml (approximately $1*10^7$ tachyzoites) of freshly lysed RHΔHXGPRT parasites were incubated in HFF cells grown in T25 flasks for 24 hours followed by one wash with PBS and a 30 min incubation at 37°C with D-MEM medium (Hyclone) supplemented with 0.1% fetal bovine serum. Subsequently ethyl methanesulfonate (EMS) (Sigma) was added to the medium at a dose of 7 mM (inducing 70% killing) and incubated for 4 hours at 37°C and 5% CO_2 followed by 3 washes with cold PBS. Host cells were scraped with a rubber policeman and then syringe passed (27G needle), filtered through a 3 μm filter, spun down and added to a fresh T25 flask confluent with HFF cells grown up at 35°C in a humidified 5% CO_2 incubator.

2.4 *Pharmaceutical Induction of Egress*

All pharmaceuticals were purchased from Sigma (except for Enhancer 2) and were diluted to working concentrations from 2 mM A23187, 1 M DTT, 2 mM Enhancer 2 (Compound ID 5137861,Chembridge), 2 mM Nigericin stocks in DMSO stored at -20°C. Concentration and exposure time for the egress inducers were as follows: 1 μM A23187 for 5 minutes, 5 mM DTT for 15 minutes, 120 μM Enhancer 2 for 30 minutes and 10 μM Nigericin for 30 minutes. Egress inducers were diluted in pre-warmed Hanks Balanced Salts Solution (HBSS) supplemented with 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM $NaHCO_3$, and 20 mM HEPES (HBSSc) [90]. Egress assays were performed at 37°C followed by fixation in 100% methanol and direct visualization by fluorescence microscopy in the case of auto-fluorescent protein expressing parasites, or by immunofluorescence using anti-SAG1 monoclonal antibody T41E5 (kindly provided by Jean-François Dubremetz)

at a dilution of 1:500 and a secondary Alexa488 conjugated goat anti-mouse antibody (Molecular Probes) at a dilution of 1:200 for non-fluorescent parasites [95].

2.5 *Induction and Reversion Egress Assay*

2F-1-YFP$_2$ and F-P2 tachyzoites were diluted to 10,000 parasites in 500 μl Ed1 medium (D-MEM (Hyclone) supplemented with 1% fetal bovine serum, 50 U/ml penicillin and 50 μg/ml, streptomycin and 0.2 mM L-glutamine) and incubated at 35°C on coverslips in a 24 well plate confluent with HFF cells. After 2 hours the wells were washed once with 1 ml PBS to remove non-invaded parasites and fresh Ed1 medium was added. One plate each for 2F-1-YFP$_2$ and F-P2 was incubated for 30 hours at 35°C and then shifted to 40°C for 0, 3, 9, 15 and 24 hours. To collect reversion kinetics, 2F-1-YFP$_2$ and F-P2 were grown for 24 hours at 40°C and then shifted to 35°C and coverslips were processed at 0, 3, 9, 15 or 24 hours post-reversion. At the indicated time points coverslips were exposed to A23187 (1 μM) for 5 minutes in HBSSc at 37°C, followed by 100% methanol fixation and mounting on microscope slides. For both induction and reversion experiments control plates for each strain were not temperature shifted and processed in parallel. Experiments were performed three times.

2.6 *Microscopy*

Microscopy was performed on a Zeiss Axiovert 200 M microscope equipped with a Plan-Fluar 100x/1.45 NA objective, a Hamamatsu C4742-95 CCD camera and the following filter sets: YFP, Q151LP-HQ500/20x-HQ535/30m; TRITC/RFP, HQ545/30x-

Q570LP – HQ620/60m; DAPI: D350/50x – E420LP – 400DCLP. Data were processed for presentation using Volocity Software (Improvision/Perkin-Elmer).

2.7 *Inhibition of Growth by Glycans*

Freshly lysed 2F-1-YFP_2 tachyzoites (2 x 10^2 parasites) were added to HFF confluent T12.5 flasks in Ed1 medium containing heparin (sodium salt), chondroitin sulfate A (CSA), chondroitin sulfate C (CSC) or dextran sulfate (DS). All chemicals were purchased from Sigma and concentrations used 0, 10, 20, 25, 35, 45 and 50 mg/ml. Plaques were stained with crystal violet after 7-9 days of growth at 37°C [92]. Experiments were independently repeated three times.

2.8 *Pyrrolidine Dithiocarbamate Killing Assays*

Freshly lysed 2F-1-YFP_2 tachyzoites (2 x 10^2) were incubated with 0, 10, 25, or 50 μM pyrroilidine dithiocarbamate (PDTC) in Ed1 medium at 37°C for 1, 2, 3, 4, or 5 hrs. The extracellular parasites were washed twice with PBS, and allowed to invade HFF confluent T25 flasks [96]. After 7-10 days of growth at 37°C plaques were stained with crystal violet [92]. Experiments were independently repeated three times.

2.9 *Procedure of the Enrichment Screen*

Ratios of RFP expressing wild-type parasites and YFP expressing F-P2 egress mutants (or 2F-1-YFP_2 wild-type parasites and TgPLP1-KO-secDsRed mutant parasites) were mixed before transfer into culture flasks confluent with HFF cells in Ed1 medium

and were incubated for 2 hours at 35°C (a total of 60,000 parasites per T12.5 for up to 1:1000 ratio; 120,000 per T25 flask for a ratio of 1:10,000 and 840,000 in a T175 flask for 1:100,000 ratio). To synchronize vacuole development, non-invaded parasites were washed away with PBS followed by addition of Ed1 medium supplemented with 25 mg/ml heparin. Flasks were subsequently transferred to 40°C for 26 hours. Flasks were then aspirated at which point 1 μM A23187 in HBSSc (pre-warmed to 37°C) supplemented with heparin (25 mg/ml) was added and incubated for 5 minutes at 37°C. Flasks were then washed once with cold PBS and subsequently Ed1 medium containing 25 mg/ml heparin and 50 μM PDTC was added and incubated for 5 hours at 35°C. Finally, flasks were washed once with PBS, fresh Ed1 medium added and flasks were incubated at 35°C until parasites lysed the HFF monolayer. YFP mutant and RFP wild-type parasite populations recovered from the screening procedure were analyzed on a Becton-Dickinson FACSAria flow cytometer (BD biosciences) within 30 minutes of harvesting. The filter set used to detect YFP was 502LP and 530/30 filters and for RFP a 556LP and 575/26 filter set was used. For the TgPLP1-KO-secDsRed versus 2F-1-YFP_2 titration enrichment experiment the screen outcome was evaluated by fluorescence microscopy since DsRed could not be detected with the filtersets available on the flow cytometer; 100 vacuoles per sample were counted.

Chapter 3. Results.

3.1 Overcoming Challenges for Development of the Enrichment Screen

The phenotypic requirements of egress mutants are unabated intracellular development whereas they should be resistant to pharmaceutically induced egress. The principle of the screen is to efficiently separate wild-type parasites sensitive to inducer-triggered egress from egress-inducer resistant parasites. Additionally, since conditional mutants would enable the most robust egress phenotypes use of temperature shifts between restrictive and permissive temperatures would be required in order to let the egress mutant phenotypes develop as well as revert back to the wild-type phenotype. Mutant phenotypes would be able to develop at the restrictive temperature (40°C) as well as allow vacuoles to grow to a size that enables it to respond to egress induction (8-16 parasites per vacuole). At the same time conditional mutants would need appropriate time to be able to recover at the permissive temperature (35°C) so that they can be cultured for further propagation.

To fulfill all the above requirements I designed the procedure outlined in Figure 6. To ensure synchronized development a population of mutagenized parasites is allowed to invade the host cells for 2 hours at the permissive temperature. At this point the monolayer is washed to remove extracellular, uninvaded parasites. Fresh medium is added and parasites are allowed to develop for 26 hours at the restrictive temperature to induce the mutant phenotypes. Subsequently egress is induced by adding the pharmaceutical of choice: wild-type parasites egress, mutants reside intracellularly. The

egressing wild-type parasites are washed away from the monolayer by a series of wash steps. Flasks are then shifted back to the permissive temperature with fresh medium to enable recovery of the mutants.

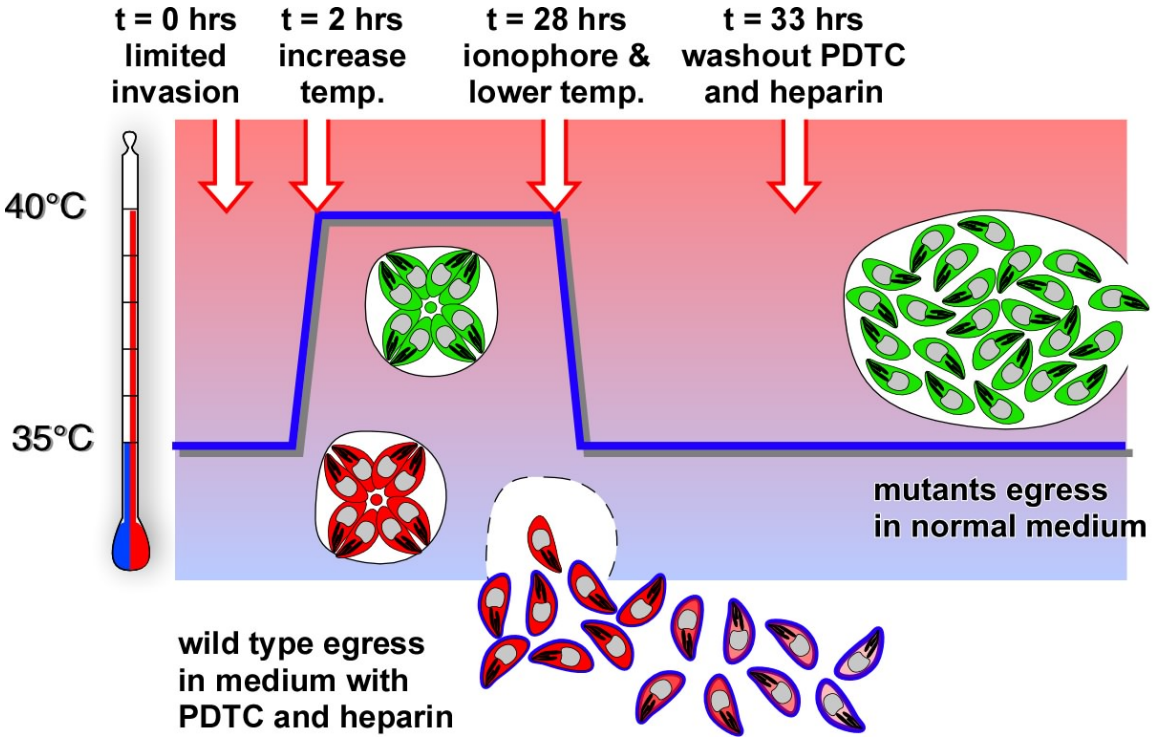

Figure 6. Schematic overview of the egress mutant enrichment screen. Temperature is indicated on the y-axis, whereas time progression is represented by the x-axis. Green parasites reflect mutant parasites, red parasites reflect wild-type parasites. The timing of changes in conditions and ionophore stimulation are indicated at the top and the blue line reflects the temperature profile. Parasites are allowed to invade at 35°C for 2 hrs to synchronize development and are subsequently shifted to 40°C to induce the egress phenotype along with 25 mg/ml heparin (preventing further invasion of extracellular tachyzoites). After 26 hrs at 40°C the parasites are stimulated with ionophore and wild-type parasites egress in medium containing 25 mg/ml heparin (preventing attachment and reinvasion) and 50µM PDTC (killing extracellular parasites). Non-egressed mutant parasites are allowed to convert back to the permissive temperature to allow recovery of viable, reversible mutants.

By generating conditional mutants, isolating egress mutants from the developed enrichment screening procedure can be followed by the new technology of cosmid

complementation of those mutants and new genes involved in egress could be characterized and the missing blanks begin to be filled in.

The envisioned screen contains two caveats; egressed wild type tachyzoites are very sticky and will resist vigorous washing and additionally other tachyzoites will within seconds re-invade upon egress. Both these events will interfere with efficient enrichment of the mutant phenotypes. To overcome these challenges the following measures were taken.

To prevent tachyzoite stickiness to the host cells I added glycans competing with the host cell attachment to the medium. It has been demonstrated before that such treatment interferes with invasion and motility of tachyzoites [21,22]. To optimize the optimal type and concentration of the previously identified glycans for the enrichment screen (dextran sulphate (DS), chondroitin sulphate A (CSA), chondroitin sulphate C (CSC) and heparin), plaque assays were set up for 9 days in the presence of various glycan concentrations to determine their inhibitory capacity. As shown in Figure 7A up to a 70% reduction in plaque number was displayed with all glycans at concentrations between 25-35 mg/ml. No plaques were observed with 35 mg/ml heparin or 45 mg/ml DS, CSA or CSC, however, upon inspection of the monolayer, at these high glycan concentrations the host cell monolayer had undergone necrosis thereby preventing plaque formation. Consequently, the 70% reduction in plaque numbers with 25 mg/ml heparin and 35 mg/ml DS are the most effective glycan conditions to prevent parasite stickiness. These conditions were used in subsequent screening procedures.

Although glycan incubation should reduce wild-type background in the enrichment by 70%, ways were explored to reduce the remaining background of 30%. The oxidizing agent pyrrolidine dithiocarbanate (PDTC) has a toxic effect on extracellular parasites but has no effect on intracellular parasites [96]. Use of this reagent would greatly reduce the remaining background in the screen, killing egressed wild-type parasites while leaving unresponsive mutant vacuoles unharmed, hence allowing a more efficient isolation of the desired mutants. Different concentrations (0-50 μM) and incubation times (0-5 hours) were tested to determine the optimal use of PDTC. No plaques formed under 25 μM for 5 hours or with 50 μM for 4 hours (Figure 7B). 50 μM for 5 hrs was selected for use in the screening procedure.

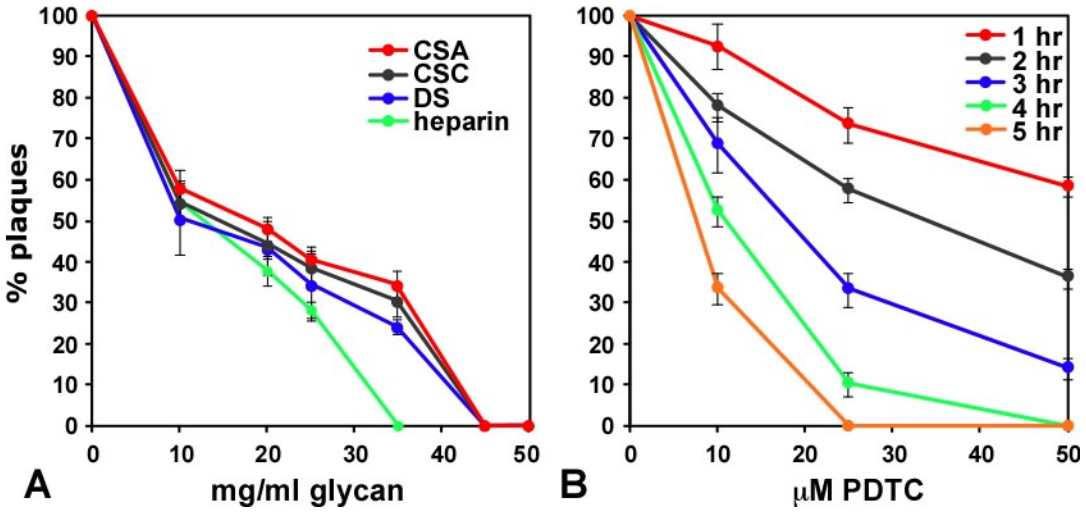

Figure 7. Assessment of various glycans and PDTC on parasite viability. (A) Plaque assays were performed on 2F-1-YFP$_2$ wild-type parasites grown for 9 days in the presence of chondroitin sulfate A (CSA), chondroitin sulfate C (CSC), dextran sulfate (DS), or heparin under the indicated concentrations. Note that the sharp drops to 0% plaques is due to killing of the fibroblast monolayer. (B) Extracellular 2F-1-YFP$_2$ wild-type parasites were exposed to PDTC concentrations of 0, 10, 25 and 50 μM for 1-5 hrs and subsequently allowed to plaque for 7 days. Plaque numbers were averaged from three independent experiments and are expressed as percent relative to the no glycan or no PDTC controls. Error bars denote standard deviation across three experiments.

An additional factor in the enrichment screen was to cope with the potential effect of the egress enhancers on the viability of parasites. For instance, the Ca^{2+} ionophore A23187 is lethal to parasites under extensive exposure [82,90]. Therefore the various pharmaceuticals inducing egress at different steps of the signal transduction pathway were tested for effects on viability of parasites. Figure 8 displays the effect of known egress inducers on parasite viability through plaque assays. Nigericin as well as ammonium chloride show a drastic detrimental toxic effect on tachyzoites. These two reagents would be unusable in the enrichment screen as all parasites subjected to the screen would be killed and hence no mutants would be recoverable. However, other well-known egress inducers such as A23187, DTT, ethanol and even small molecule microneme enhancers [88] have a manageable effect on parasite viability.

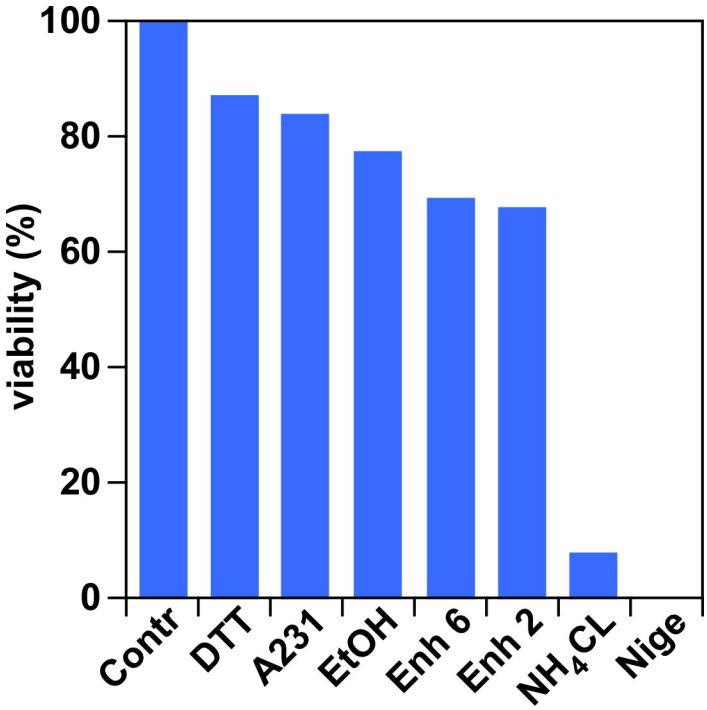

Figure 8. Evaluation of recovery from exposure to egress inducers. The plaque forming capacity of induced mutant F-P2 upon return to the permissive temperature following exposure to various inducers was tested as a measure of viability to determine whether egress mutants would be recoverable. DTT, A23187, ethanol, small molecule microneme enhancers 2 and 6 [88] have little effect on parasite viability. Nigericin and ammonium chloride have a toxic effect on parasite viability.

3.2 Testing Enrichment Power Utilizing Egress Mutant F-P2

By running pilot screens by diluting an egress mutant in hand into a pool of wild-type parasites I would be able to assess the specificity and the enrichment power of the designed screen. An egress mutant fulfilling the requirements of the screen was identified in a screen for general defects in the lytic cycle [14]. This mutant, named F-P2, was generated by chemical mutagenesis and displays a temperature sensitive growth defect. As shown in Figure 9, F-P2 grows normally for the first 3 days but arrests after this time. Upon a detailed microscopic inspection mutant F-P2 replicates just as wild-type, but fails to egress from its vacuole at the restrictive temperature.

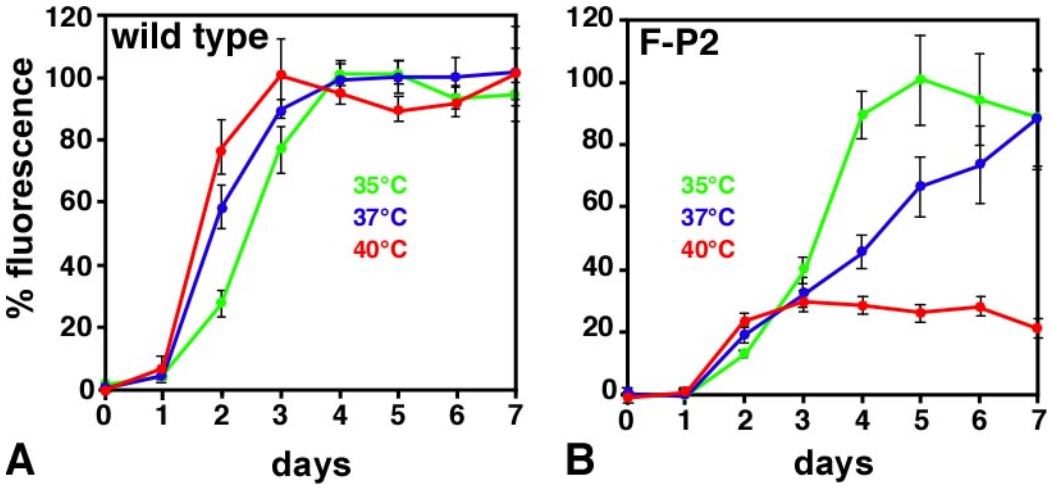

Figure 9. Temperature sensitive mutant F-P2 has a growth defect after 3 days at 40°C. Temperature sensitive mutant F-P2 is a growth mutant. Growth curves of parent 2F-1-YFP_2 (A) and temperature sensitive mutant F-P2 (B) were collected in 384-well plates using the level of YFP expression as read-out [93]. Error bars represent standard deviation of quadruple wells.

F-P2 was resistant to A23187 induced egress at 40°C whereas at 35°C F-P2 responds to ionophore by egress from the host cell indistinguishable from the wild-type response (Figure 10A-D). To better understand the kinetics of this phenotype, induction and reversion experiments were performed. For induction of the phenotype at the restrictive temperature, the mutants progressively lost their capacity to respond to egress induction. After 15 hours of induction, vacuoles became completely resistant to Ca^{2+} ionophore treatment (Figure 10E). Upon reversion of the phenotype at the permissive temperature, the F-P2 mutant continually re-gains its ability to respond to Ca^{2+} ionophore. At 15 hours post-reversion F-P2 is responsive to Ca^{2+} ionophore (Figure 10F).

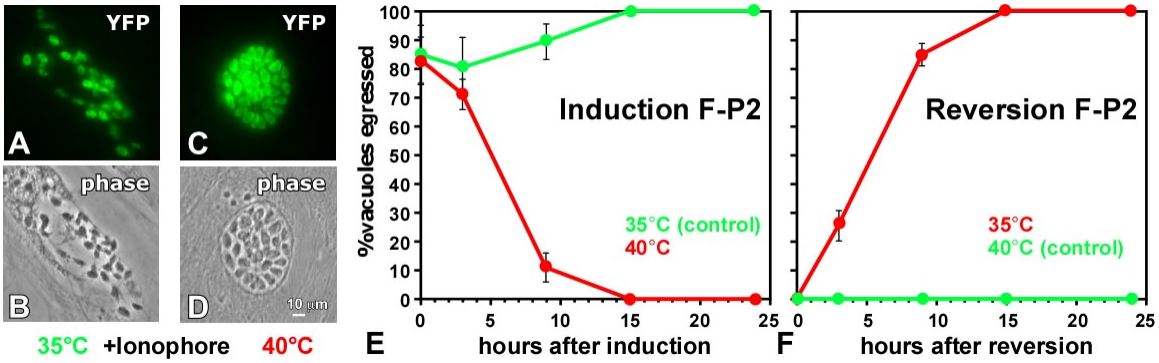

Figure 10. Temperature sensitive mutant F-P2 has a reversible egress defect. Parasites were grown at either 35°C (permissive temperature) or 40°C (restrictive temperature), as indicated. (A,B) F-P2 parasites grown for 36 hrs at 35°C and stimulated with 1 µM Ca^{2+} ionophore (A23187) for 5 minutes do egress normally, leading to vacuole rupture and parasite dispersal. (C,D) F-P2 parasites grown for 36 hours at 40°C and stimulated with 1 µM A2387 do not egress, leaving the vacuolar membrane intact. (E) Kinetics of F-P2 phenotype induction at 40°C. F-P2 parasites were grown for 30 hrs at 35°C and transferred to 40°C (red line) or kept at 35°C (green line, control) at t = 0 hrs. Parasites were stimulated with 1 µM A2387 at indicated time points and vacuoles scored for egress by microscopy. After 15 hrs 100% of vacuoles lose the ability to egress. (F) Kinetics of F-P2 phenotype reversion after switching fully induced parasites (24 hrs at 40°C) back to 35°C (t = 0 hrs) and measuring 1 µM A2387 stimulates egress at indicated time points. All vacuoles convert back to a fully A23187 responsive phenotype after 15 hrs (green line) whereas the control kept at 40°C does not convert (red line). Averages of three independent experiments are shown; error bars denote standard deviation.

Wild-type parasites are responsive to A23187 upon both induction and reversion temperature shifts, in contrast to the F-P2 mutant (Figure 11). Since F-P2 displays a reversible egress defect and contains no growth defect, it perfectly fits into the envisioned screen format in Figure 6.

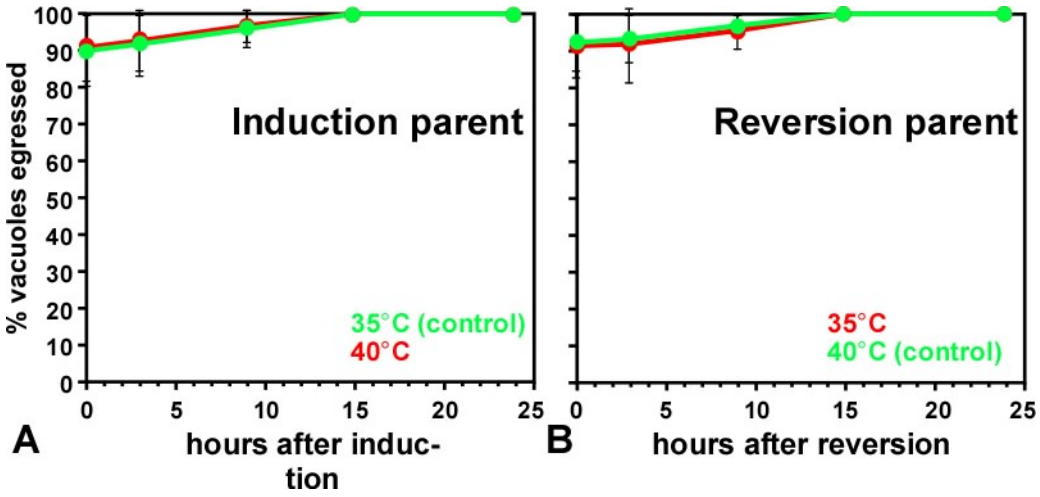

Figure 11. Wild-type 2F-1-YFP$_2$ parasites are fully responsive to A23187 regardless of temperature. Wild-type parent control assays for the kinetics of the reversible stimulated egress defect shown for F-P2 mutant in Fig. 10E and F. (A) Parasites were grown for 30 hrs at 35°C and transferred to 40°C (red line) or kept at 35°C (green line, control) at *t* = 0 hrs. Parasites were stimulated with A23187 at indicated time points and vacuoles scored for egress by microscopy. No significant differences between the two temperatures are observed. (B) At *t* = 0 hrs, parasites grown for 24 h at 40°C are placed back to 35°C (red line) or kept at 40°C (red line, control). A23187 stimulated egress was measured at indicated time points. No significant differences between the two conditions are observed. Averages of three independent experiments are shown; error bars denote standard deviation.

Conveniently, F-P2 expresses a cytoplasmic fluorescent reporter (YFP) that is easily measurable by flow cytometry. In order to reliably distinguish F-P2 mutants from wild-type parasites, I used wild-type parasites expressing cytoplasmic red fluorescent protein (TomatoRFP), which can similarly be detected by flow cytometry (Figure 12A,B). Ratios of YFP expressing F-P2 mutants mixed with wild-type expressing RFP parasites in ratios ranging from 1:1 to 1:100,000 were used to assess the enrichment power of the screen. Viability of each strain was assessed by plaque assays in order to account for differences in viability between these two strains. At ratios of $1:10^2$ there was 100% recovery of the mutant phenotype, at $1:10^3$ there was 98% recovery and at ratios of

1:10^4 there still was 60-80% F-P2 recovery. At 1:10^5 recovery drops into single digits following just one round of screening (Figure 12C).

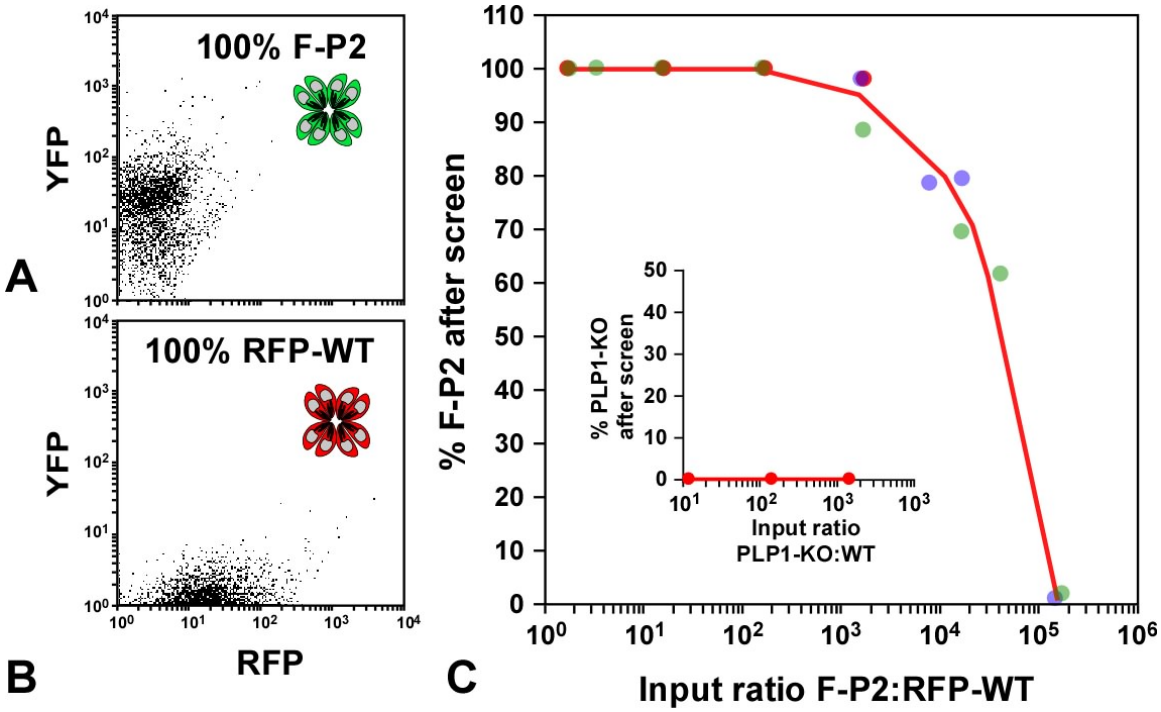

Figure 12. Assessment of the power of the screen to enrich for egress mutants. Cytoplasmic YFP expressing F-P2 mutant parasites were mixed with cytoplasmic RFP expressing wild-type parasites at different ratios and subjected to the screen in Figure 6. Recovered parasites were allowed to grow for 7 days and were analyzed for their fluorescent signature to differentiate wild-type from mutant parasites by flow cytometry. (A,B) Cytometry profiles of unmixed populations of F-P2 (YFP expressing) and wild-type parasites (RFP expressing) display good separation in the fluorescent channels on the flow cytometer. (C) Enrichment results of the F-P2 mutant phenotype from mixtures of wild-type and mutant parasites. Data points from three independent experiments are shown in red, blue and green. Insert in C. Enrichment results of the TgPLP1-KO mutant expressing vacuolar RFP mixed with wild-type parasites expressing YFP scored by fluorescence microscopy. Ratios are corrected for differences in viability of wild-type and mutant parasites as assessed by plaque assays (not shown).

An additional egress mutant was found in the TgPLP1-KO mutant. This mutant is still motile but fails to secrete the perforin-like protein (TgPLP1) required for escape from the vacuole and host cell. However, this mutant is still able to egress by mechanical force that the motility of the parasite generates on the vacuolar membrane resulting in a 10-15 minute delay in egress compared to wild type parasites in response to Ca^{2+} ionophore

treatment. A DsRed fluorescent version of this mutant was utilized to assess the capability of the screen to enrich for its delayed egress phenotype. In this case wild-type expressing YFP and TgPLP1-KO DsRed RFP mutant parasites were mixed at various ratios and subjected to the screen. For this experiment enrichment was evaluated by microscopy since DsRed could not be detected by the flow cytometer. In all the tested ratios no enrichment of the TgPLP1-KO mutant was detected (Figure 12C insert).

3.3 Isolation of DTT Resistant Chemical Mutants

Since the NTPase mediated pathway is the least understood egress pathway, I mutagenized parasites and screened for DTT resistant egress mutants. Assuming that this pathway would involve essential genes, chemical mutagenesis using EMS was used to establish a pool of mutants. In addition to screening for DTT resistant mutants EMS mutagenesis was also applied to generate and isolate A23187 resistant mutants. Three independent chemically mutagenized pools generated by EMS mutagenesis were subjected to two subsequent rounds of enrichment screening for resistance to DTT and A23187 separately. A typical population complexity is in the order of 10^4-10^5 from a 70% killing dose [14]. With our enrichment power we would expect the mutant recovery to be in the single percent range after one round of screen enrichment. Upon a second round of screening any mutants of interest in the mutagenized pool of parasites would be easily recovered. In all three screens, DTT resistant mutants were isolated. However, we were unable to isolate any A23187 resistant mutants. Enriched parasites were cloned by limiting dilution and one DTT mutant per mutagenesis was further studied. I named

these mutants based on the EMS mutagenesis number, the enhancer with which the mutant was isolated followed by the final clone number (e.g. EMS2DDT2 is EMS mutagenesis 2, DTT enrichment screen, clone2, as well as EMS3DTT3, EMS4DTT5, respectively).

Characterization of the phenotype by evaluation of the egress response to different inducers in addition to DTT showed the mutants were cross-resistant to small molecule Enhancer 2 and Nigericin. However the mutants were fully sensitive to A23187 induced egress. Upon assessment of the temperature-sensitive nature of the mutation we discovered that the DTT mutants were also resistant to induced egress at the permissive temperature. Therefore, the phenotype is not temperature sensitive, which suggests that the mutation is not in an essential gene (Figure 13). Since the phenotype of these mutants is constitutive and has no difference at 35°C and 40°C, complementation efforts of these phenotypes would be complicated due to the background of egressed parasites. To this end, I chose to test the phenotype of these mutants at 34°C and 41.5°C in order to achieve a 0% egress background for future complementation efforts. The intended goal was that at 41.5°C there would be a 0% background.

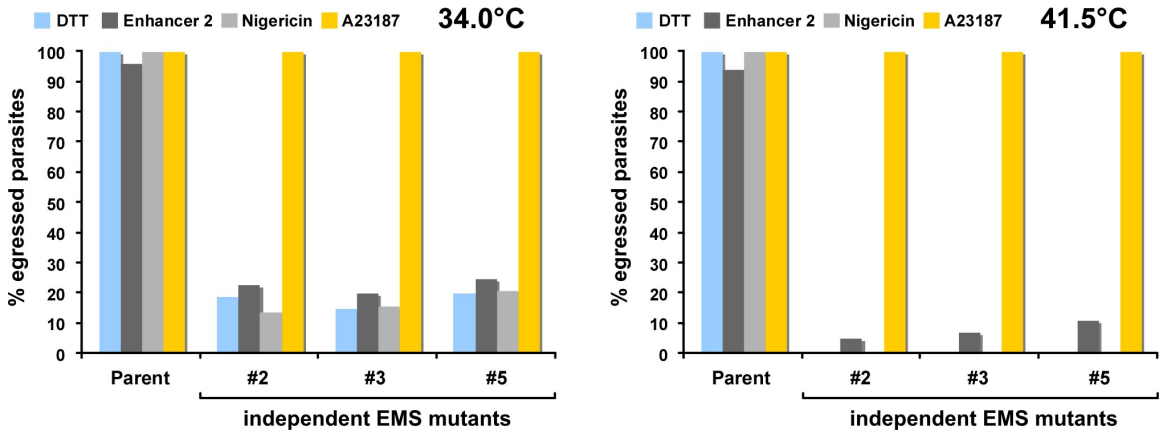

Figure 13. Three generated and isolated EMS mutants have constitutive egress defects.
Three generated and isolated EMS egress mutants utilizing the screen outlined in Figure 6 were tested for their ability to egress in response to egress inducers. The egress mutants have a constitutive egress defect in response to DTT, nigericin, small molecule microneme enhancer 2. The egress mutants are fully sensitive to the Ca^{2+} ionophore A23187.

3.4 Isolation of a DTT Resistant Insertional Mutant

The fact that the DTT mutants generated by chemical mutagenesis display a constitutive phenotype suggests that such mutants can also be generated by insertional mutagenesis. To test this hypothesis I generated a pool of insertional mutants using the ToxoSuperCos plasmid endowing pyrimethamine resistance to successfully transfected parasites. Following two rounds of enrichment through the screen using DTT as the egress enhancer we were indeed able to isolate a DTT resistant mutant (mut-IM-DTT). Functional analysis of mut-IM-DTT showed that it is fully responsive to A23187, but has an approximate 70% egress defect in response to DTT (Figure 14). As a control, F-P2 is fully responsive to both inducers at 35°C, but is resistant to both DTT and A23187 at 40°C.

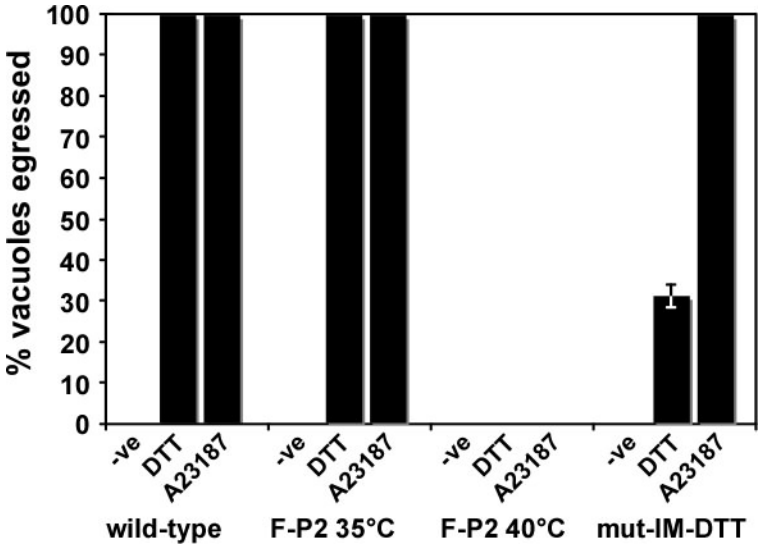

Figure 14. Egress assays using DTT and A23187 as egress enhancers on wild-type, mutant F-P2 and mut-IM-DTT. Mutant mut-IM-DTT was generated by insertional mutagenesis and enriched using the procedure outlined in Figure 6 using DTT instead of A23187 as an egress enhancer. Wild-type parasite egress is identical at 35, 37, and 40°C (not shown). Mut-IM-DTT was evaluated at 37°C. Intracellular versus egressed vacuoles were counted and plotted as the percentage of total vacuoles. Data represent three independent experimental replicates; error bars denote standard deviation.

Chapter 4. Discussion.

The objective of this thesis was to develop and implement a novel enrichment screen that would enable the efficient isolation of egress mutants with defects in distinct steps. The basis for the screen is the efficient separation of intracellular from extracellular parasites, which were major challenges of a screen with the same goal in the past [90]. The innovative aspects of the screen are the combinatorial use of 25 mg/ml heparin, which prevents attachment and reinvasion, and 50 μM PDTC, which specifically kills extracellular parasites (Figure 7A,B). The glycan optimization results are in agreement with the data reported in previous studies looking at inhibition of invasion and gliding motility by the same glycans by binding to host cell surface receptors as well as to the parasite [21,22]. The toxic effects of measured viability for PDTC treatment on extracellular parasites were also in agreement with previous work, albeit that the length of incubation times was extended in this study to ensure maximal selection [96]. Through the use of these reagents the biggest hurdle of the screen was overcome. Plaque assays were chosen to assess the effectiveness of these reagents, as visible plaques in a monolayer are evidence of completed rounds of the lytic cycle directly involving invasion as well as the overall viability of a parasite.

In order to assess enrichment power of the screen, two available mutants were utilized. The first mutant F-P2 is a conditionally lethal mutant that has a natural egress defect. Additionally, F-P2 has a completely reversible egress defect in response to Ca^{2+} ionophore as long as the parasites remain intracellular (Figure 10). This particular mutant

has the exact conditional reversible phenotype that is required to be isolated from the enrichment screen. Mixture of mutant with wild-type parasites showed that F-P2 could be routinely enriched 1000-fold from a pool of wild-types (Figure 12). From a typical insertional mutagenesis event the number of independent transfectants is in the order of 10^4 [15], for a typical chemical mutagenesis the complexity of a population using a 70% killing dose is in the order of 10^4-10^5 [14]. Whichever type of mutagenesis is performed, the enrichment capacity to be able to isolate egress mutants is well within the power of the screen. Since after a single round of screening with a ratio of 1:100,000 the enrichment power is in the single digits for recovery of the conditional lethal mutant, two rounds of screening are needed to isolate and recover any mutants generated.

In addition to this conditional lethal mutant, a mutant with a delayed egress defect was tested. This mutant is a knock-out in the microneme secreted perforin TgPLP1, and in contrast to mutant F-P2, is fully motile. As a result the TgPLP1-KO mutant egresses by mechanical force over a time period of 10-15 minutes rather than seconds observed in wild type parasites in response to Ca^{2+} ionophore treatment [63]. I was unable to enrich the TgPLP1-KO parasites from a pool of wild-type parasites showing the limitation of the screen (Figure 12C insert). This is much shorter of a time frame than the 5 hour period that the screen contains to rid the population of extracellular parasites. Delayed egress phenotypes as seen in the TgPLP1-KO will therefore not be among the expected phenotypes of mutants enriched employing the screen.

Since the screen demonstrated robust enrichment for conditional lethal phenotypes displaying Ca^{2+} ionophore resistance, within the expected population

complexities of both chemical and insertional mutagenesis, the screen was used to detect defects upstream of the Ca^{2+} release such as the response to DTT as well as defects downstream or at the Ca^{2+} release in response to A23187. Three independent, chemically induced, constitutive egress mutants resistant to DTT were isolated, which were cross-resistant to Nigericin and small molecule microneme Enhancer 2 (Figure 13). This cross-resistance would indicate that the defect in these mutants lies at or after the PLC defect but before the actual release of Ca^{2+}. However, considering our model of signaling in egress (Figure 5) it should be possible to generate mutants resistant to DTT and sensitive to ethanol. In future screens we will therefore combine subsequent positive and negative screening using DTT and ethanol, respectively.

The three DTT-resistant mutants generated by chemical mutagenesis were surprisingly not temperature sensitive. The ability to isolate such constitutive egress mutants suggests that the mutated gene is not an essential one. Hence, it must be possible to isolate mutants with a similar phenotype by insertional mutagenesis. One independent insertional mutant was isolated through the enrichment screen in response to DTT. The insertional mutant (mut-IM-DTT) egress phenotype displayed a 70% reduction in DTT induced egress and was found to be fully responsive to A23187 (Figure 14).

Efforts to genetically complement the chemically induced mutants through cosmid complementation were performed. The goal was to restore the wild-type phenotype to 100%. Following this complete phenotype restoration, plasmid rescue followed by individual gene complementation would enable the identification of the mutated genes resulting in the mutant phenotypes [14]. Despite the proven high rate of

successful complementation of mutant phenotypes I was unable to achieve a 100% wild-type phenotype restoration with any of the three mutants. For mutant EMS4DTT5 I was able to only partially restore the phenotype of this mutant through cosmid complementation. The rescued region from plasmid rescue resulted in Chromosome X, nucleotide position 4,046,932-4,096,932. Six predicted genes were in this region including 28.m00287, 28.m00548, 28.m00286, TgGlmHMM_2655, 28.m00285 and 28.m00284. All predicted genes are hypothetical except for 28.m00286 which has a kinesin motor domain. However, individual gene complementation resulted in no restoration of the mutant phenotype at all. Hence, the mutated genes causing these mutant phenotypes remain unknown in all three mutants.

Theoretically, it should be easier to map the genetic defect underlying insertional mutants than for chemical mutants [15]. To this end extensive efforts were made to rescue the inserted piece of the transfected linearized vector along with a piece of the flanking genomic DNA in our insertional mutant. This would enable inserted gene identification resulting in the mutant phenotype. Despite many attempts I was unable to rescue the insert along with a flanking piece of genomic DNA.

In-spite of the fact that the identification of the underlying mutated genes for each of the four egress mutants isolated remain unknown, development and utilization of the enrichment screen described herein is impactful for the field for time to come. In addition, the non-conditional nature of the chemical as well insertional mutants resistant to DTT indicates that there must be another pathway that results in normal egress in these mutants. Since we are unable to directly test ABA induced egress due to its low induction

efficiency (7%) [11], it is likely that this pathway is not mediated by the gene(s) mutated in the present mutants. Therefore, just by assessing the nature of the mutants, the connections between the signaling pathways can already be mapped out, even in absence of the identity of the genes underlying these phenotypes.

We were unable to isolate any A23187 resistant egress mutants with a similar phenotype as the F-P2 conditional mutant even when two rounds of enrichment screening were applied. However, this is not a complete surprise as the F-P2 mutant has resisted genetic complementation leading to the assumption that F-P2 contains multiple mutations that could be responsible for the conditional phenotype. It is therefore conceivable that pathways downstream of Ca^{2+} are redundant and would require two independent mutations, which will be extremely rare, with mutant F-P2 as the exceedingly rare exception.

Interestingly, the Type II Pruginaud strain has a natural resistance to the K^{+} ionphore Nigericin (Gustavo Arrizabalaga, personal communication and results not shown). It would be interesting to utilize the enrichment screen to rescue this naturally occurring mutant phenotype through cosmid complementation in response to a potassium ionophore that is not as toxic to the parasites as Nigericin is. In this specific scenario egressed parasites that behave as wild-type would be the parasites to recover.

Upstream defects in parallel pathways converging to calcium release can be dissected by insertional mutagenesis through the use of diverse egress inducers. Downstream defects in egress can in theory also be genetically dissected by generating conditional mutants through chemical mutagenesis, assuming the downstream pathways

are non-redundant. Additionally, this screen could be applied to dissecting immune response induced egress as well (e.g. T cell mediated) [62].

The developed and employed enrichment screen described herein is a new, powerful and versatile tool to genetically dissect *Toxoplasma* egress. I anticipate this screen will lead to the identification of various new genes with roles in egress and help fill in the current paucity in molecular players fitting the pharmaceutical data. Taken together, this will lead to better elucidated pathways and will highlight specific candidate targets for drug therapies. Such leads can then be developed into useable drugs to combat the destructive effects of not only the lytic cycle of *Toxoplasma gondii*, but potentially other diseases caused by apicomplexan parasites as well, due to the conserved nature of many aspects of the invasion and egress pathways across the phylum.

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